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Physical interaction between the invertebrate photoreceptor-specific arrestin homolog, phosrestin-I, and myosin V in head of Drosophila melanogaster

Erik D. Weiss
Yale University

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Physical interaction between the invertebrate photoreceptor-specific arrestin homolog, phosrestin-I, and myosin V in head of *Drosophila melanogaster*

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Erik D. Weiss

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Abstract:

Myosins are a superfamily of actin-based ATPase molecular motors that use actin filaments as tracks for unidirectional force production. In addition to conventional myosin's traditional role in muscle contraction, myosins are involved in a variety of cellular functions including cell polarity, membrane trafficking, and signal transduction. Class V myosin is an unconventional myosin identified in a wide variety of species, suggesting its conserved importance in the cytoskeletal functions of higher eukaryotes. We sought to identify binding partners for myosin V in the head of *Drosophila melanogaster* in order to elucidate possible roles for this molecular motor in this invertebrate species. A possible role for myosin V in photoreceptors of the fruit fly has emerged from our finding that myosin V copurifies with the arrestin homolog, phosrestin-I, a photoreceptor-specific protein involved in light inactivation. We utilized a baculovirus-expressed recombinant myosin V protein and a FLAG tag-based affinity purification technique to draw putative binding partners from a *Drosophila* head extract and size fractionated proteins on SDS-PAGE as well as confirmed myosin V presence by Western blotting with myosin V polyclonal antibodies as well as a monoclonal antibody for the FLAG peptide. Protein bands did not appear in experiments conducted without recombinant myosin V and *Drosophila* heads. The resulting coomassie-stained polyacrylamide gel was analyzed by liquid chromatography-mass spectrometry (LC-MS) (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH). The rationale for our finding is supported by an earlier demonstration that myosin V localized to the rod photoreceptor cells of the rat retina. Myosin V has varied roles in intracellular trafficking. It is possible that this actin-based cytoskeletal molecular motor may have a role in the localization and interactions of phosrestin-I in the process of light inactivation in *Drosophila* photoreceptors in response to light activation of rhodopsin and the resulting G protein-coupled signaling cascade and Ca^{2+} influx.

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CONTENTS

INTRODUCTION

Myosins are a superfamily of molecular motors that use actin filaments as tracks for unidirectional force production. Myosins are defined by a conserved motor domain, which consists of an amino-terminal globular head. The globular head contains nucleotide- and actin-binding sites, is generally built from 720-780 amino acid residues, and hydrolyzes MgATP in an F-actin-regulated fashion (Figure 1). Myosin, the thick filament in muscle contraction, is capable of translocating the thin actin filaments to produce motor function or to transport vesicles or other cargo in the cytoskeleton. Myosins are grouped into classes, currently 16, based on sequence homology of their head domain sequences [1][2].

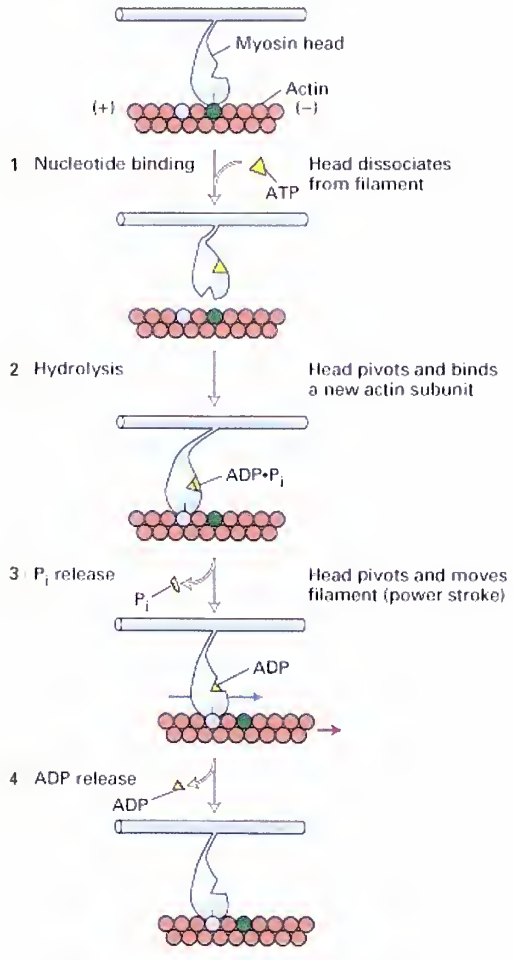


Figure 1. Actin-myosin coupling [3]

Myosins generate energy by hydrolyzing MgATP. The MgATPase activity of myosin at baseline is generally quite low and only increases upon interaction with actin. In some myosin classes, such as myosin II from vertebrate skeletal muscle, the actin-dependent MgATPase activity is regulated by the troponin-tropomyosin system. In other myosin classes, the MgATPase activity is governed by phosphorylation or by the flux of calcium ions in the environment [2].

Attached to its one- or two-headed motor domain, myosins have a neck region consisting of a long, single α-helical strand from the heavy chain supported by the binding of light-chain subunits. The myosin neck domain contains 1-7 light-chain binding regions, known as IQ motifs due to their conserved isoleucine and glutamine residues. These light chains are members of the calmodulin/EF-hand superfamily of calcium-binding proteins. Light chains provide rigidity to the neck region, may function in extending the length of the myosin head for optimization of lever-arm force production, and regulate myosin's motor activity by binding Ca^{++} [1] [2].

The tail region, which is thought to determine the interactions and localization of the myosin, and therefore much of its function, varies widely between the myosin classes. Some myosins have tail domains that dimerize through the formation of coiled-coil structures and thus assemble to form filaments. These are known as conventional myosins (ie. myosin II found in skeletal muscle and cytoplasmic myosins from unicellular organisms). Only the well-known muscle myosin II is capable of forming thick filaments at low ionic strength that pull along thin actin filaments in the process of muscle contraction. The myosins that cannot form filaments are known as unconventional myosins and carry calmodulin molecules as light chains [4]. Regardless

of its ability to dimerize and to form filaments, the tail region anchors the myosin so that it can move relative to actin [1][2].

MYOSIN V

Vesicles and organelles in the axoplasm of the squid giant axon move along actin filaments in association with a motor protein bearing a head domain consistent with the myosin superfamily. This molecular motor was first isolated as a calmodulin-binding protein from brain. Class V myosin has emerged as the prime candidate for vesicle/organelle transport. Myosin Va purified from chick brain translocates beads along actin filaments *in vitro* [1][5].

Conventional myosin spends only ~5% of its mechanochemical cycle attached firmly to actin filaments. However, transport of organelles or vesicles by a myosin moving along actin requires that the motor move a long distance along the filament before detaching. Myosin V binds more tightly to actin filaments in the presence of MgATP than does conventional myosin, indicating that it could be bound to actin for a longer fraction of the cycle time, and thus perhaps move a longer distance before detaching [1]. High-affinity binding of myosin V to actin is calcium dependent. In the presence of calcium and MgATP, the high affinity binding to actin is a property that distinguishes the class V myosins from other myosin classes. Such high-affinity binding to its filamentous track is a characteristic of a processive motor. Processive motors undergo multiple cycles of MgATP hydrolysis with movement along a filament before dissociation. Myosin V is the first example of an actin-based processive motor and moves actin at approximately 400 nm/s [5].

Currently, nine complete myosin V heavy chain sequences have been identified. *Saccharomyces cerevisiae* has two myosin V's, *Caenorhabditis elegans* has one, *Drosophila melanogaster* has one, and vertebrate species have at least three differentially expressed subclasses [5].

Purified brain myosin V (B-MV) exists as a two-headed dimer[1]. Brain myosin V is an oligomeric molecule with two identical heavy chains of 212 kDa. It has 12-14 light chains (10-20-kDa), including at least 8 calmodulin molecules comprising the major light chains [4].

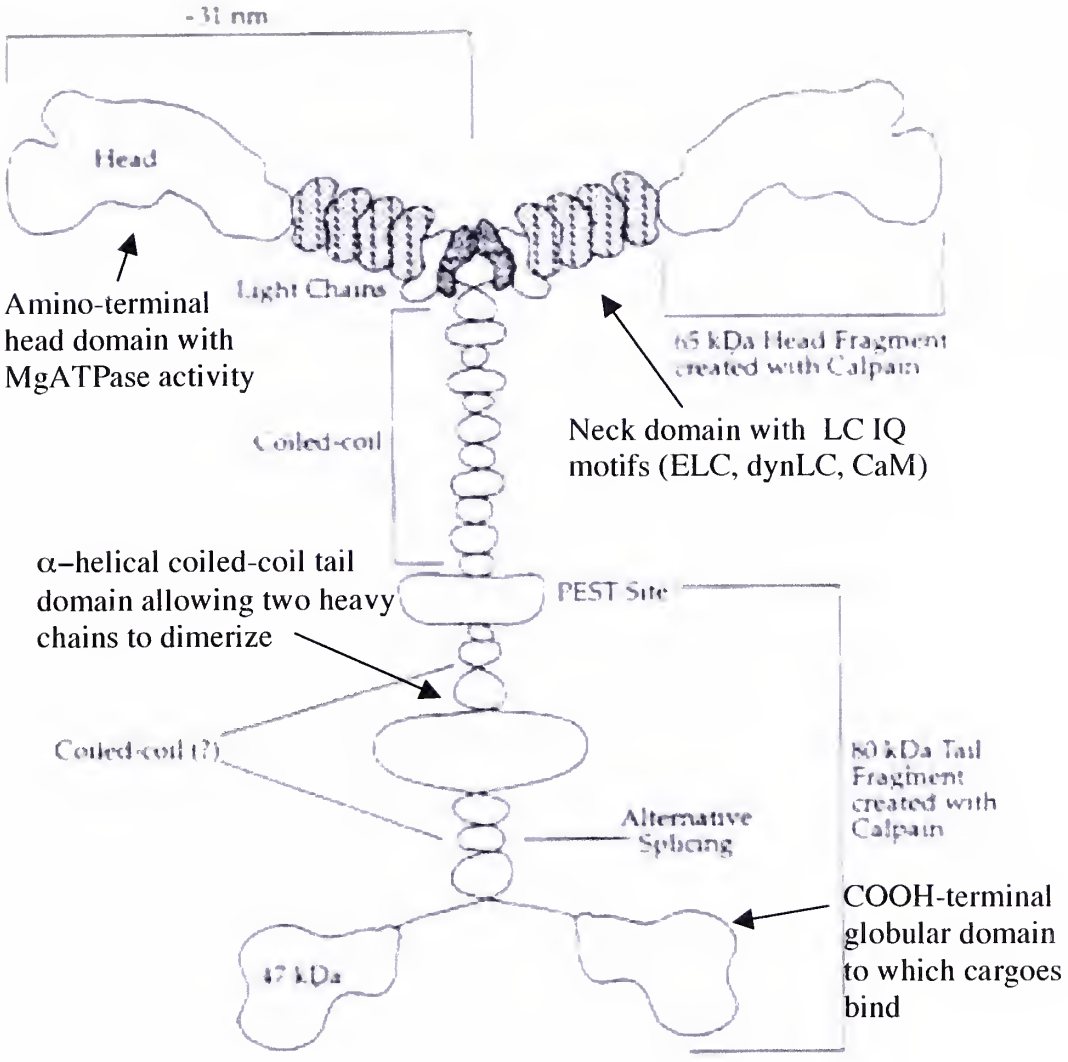


Figure 2. Myosin V [6]

The head/motor domain is the most amino-terminal region of the myosin V protein. The neck domain contains the putative essential light chain (ELC) and calmodulin (CaM) binding sites. The tail region is divided into a domain predicted to form an α-helical coiled-coil structure and a carboxyl (COOH)-terminal globular domain. The motor domain and first IQ motif of the neck are well conserved among myosin V subclasses, while the globular tail is divergent [5] (Figure 2).

The MgATPase of chick brain myosin Va is activated by actin in a calcium-dependent fashion, as is that of most myosins. In the absence of calcium, actin has no

effect on MgATPase activity. At calcium concentrations of ~3 μM, the MgATPase activity of chick brain myosin Va increases to as high as 30-50 ATP molecules/seconds/head in the presence of actin. Since actin activation of the MgATPase of baculovirus-expressed tail-less myosin Va is calcium independent, as is a motor domain separated from the tail domain, the regulation of myosin Va MgATPase by calcium may require molecular interactions, perhaps calmodulin-mediated, between the head, neck, and tail of the myosin molecule. High concentrations (~15 μM) of calcium inhibit myosin V-based motility in *in vitro* motility assays, perhaps due to calcium-dependent calmodulin dissociation, an effect ameliorated by addition of exogenous calmodulin [5][2].

The neck domain of myosin V contains six light chain (LC) binding motifs. These have a consensus sequence of IQXXXRGXXXR, which serves as a binding site for calmodulin and for myosin light chains of the EF-hand superfamily of calcium-binding proteins. Biochemically purified chicken myosin Va has been shown to co-purify with ~5 calmodulin molecules per heavy chain. In general, myosin Va co-purifies with light chains including the 17- and 23-kDa essential light chains. Chicken myosin Va also co-purifies with an 8 kDa light chain from the microtubule motor dynein, the dynein light chain (DLC, actual molecular weight 10 kDa) with 2 DLCs per myosin V dimer. This DLC is highly conserved in diverse species including *Chlamydomonas*, *S. cerevisiae*, *Drosophila*, *Aspergillus*, *Schistosoma mansoni*, and *Homo sapiens*. The DLC may stabilize heavy chain-heavy chain interactions, act in vesicle or organelle cargo binding, and interact *in vitro* with nitric oxide synthase (NOS) and inhibitor of NF-κB (IκB). The calcium regulation of myosin V MgATPase rate indicates that light chains may have

additional regulatory roles in higher eukaryotes, in addition to conserved functions in stabilizing the motor lever arm. Calcium regulation could mediate changes in calmodulin binding to the neck domain [5][7].

The small size, high solubility, and abundance of calmodulin suggests a general access of calmodulin to its targets. However, calmodulin specifically localizes in particular subcellular regions, including postsynaptic densities, centrosomes, the mitotic apparatus, the contractile vacuole of Dictyostelium, and the growing bud and cytokinesis contractile ring of yeast. The interaction of calmodulin with the unconventional myosin motor proteins may make these localizations possible [4].

The neck-tail junction of myosin V is not well conserved, indicating that it may have diverged to achieve specific motor-neck or motor-cargo interactions in a species-specific fashion. The tail domain consists of an α-helical coiled-coil region of variable length, which allows the two heavy chains to dimerize, followed by a COOH-terminal globular domain which is possibly involved in cargo binding and/or intracellular localization. With respect to possible localization functions of the tail, GFP-tail domain constructs have been shown to co-localize with melanosomes and centrosomes, both locations of myosin V staining. In yeast, Myo2p localizes to the bud tip of dividing cells and yeast in which the tail domain of Myo2p has been deleted are not viable [5][2].

The AF-6/*canoe* family of proteins has homology to the myosin V tail domain. AF-6 was first identified as an ALL-1 fusion partner in some patients with acute lymphoblastic leukemia. In *Drosophila*, *canoe* interacts genetically with the *Notch* and *Ras* signaling pathways. The *C. elegans* AF-6 homolog also interacts with *Ras* signaling.

Thus, the AF-6 homology domain on the myosin V tail may be a link between the cytoskeleton and cell signaling cascades [5].

MYOSIN V FUNCTIONS

Studies of intracellular localization and the phenotypes of null mutations in mouse and yeast indicate that myosin V may serve a role in vesicle movement *in vivo*. Class V myosin localizes to synaptic vesicles, where it can interact with the synaptophysin-synaptotagmin complex involved in SNARE regulation and internal membrane fusion in eukaryotes. Synapses of rod photoreceptors in rats and neuronal cells of the cochlea express detectable amounts of myosin V, as do melanosomes [2].

In mice, myosin Va heavy chain is the product of the *dilute* gene, associated with dilute or pale coat color. Normally, pigment is synthesized by melanocytes in melanosomes, passed through melanocytic dendritic processes, and delivered to keratinocytes.

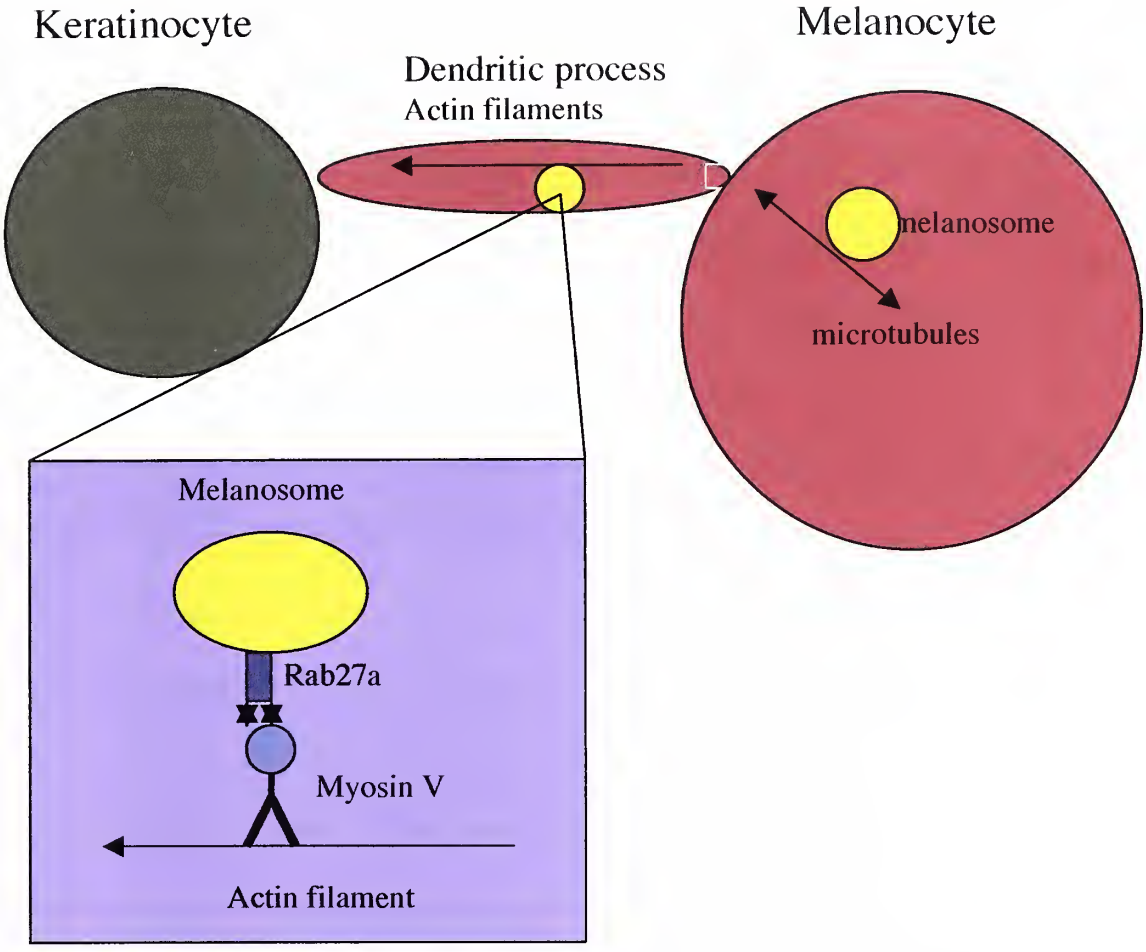


Figure 3. Myosin Va and Rab27a are involved in the capture of melanosomes in melanocytic dendritic processes.

The dilute color of these mice is due to defective transfer of melanin from melanocytes to the hair shaft [1]. In order to accomplish the transfer of melanosomes from melanocytes to keratinocytes, melanosomes are first accumulated at the distal ends of the melanocytic dendritic extensions. The movement of melanosomes is achieved by a cooperative transport mechanism involving central, fast, long-range, bidirectional, microtubule-dependent movement and myosin-Va-dependent capture and local movement along distal, actin-based dendrites (Figure 3). In *dilute* mice, this capture mechanism is missing. The melanosome capture mechanism requires both myosin Va and a member of the Rab

family of small GTPases, Rab27a. This Rab family member enables myosin Va-dependent melanosome capture and recruits the myosin motor to the melanosome surface, perhaps acting at least in part as the melanosome receptor for myosin Va [8].

Some of the alleles of *dilute* also are associated with neurological defects. Mice bearing two null alleles of the gene suffer fatal seizures early in life, suggesting a role for myosin V in neurosecretion [2]. These diverse phenotypes are consistent with the human myosin V mutation seen in Griscelli disease. The Griscelli G5 locus colocalizes on chromosome 15q21 with the myosin Va gene (MYO5a). Griscelli disease is an autosomal recessive condition characterized by pigmentary dilution, silvery hair, pigment aggregates in the hair shafts, and accumulation of melanosomes in melanocytes. These patients also suffer from variable cellular immunodeficiency involving absent delayed-type cutaneous hypersensitivity and impaired T-cell and natural killer (NK)-cell function. The disease is associated with hemophagocytosis, leading to acute phases of lymphocyte and macrophage activation resulting in fever, hepatosplenomegaly, CNS infiltration by lymphocytes and macrophages, coagulopathy and pancytopenia that occur by a mean age of 36 months. Without a bone marrow transplantation, death occurs by a mean age of 5 years [9][10].

PROJECT PURPOSE

Studies exploring the actions of myosin V in humans and other species have demonstrated its importance in neurological development and function. Our hypothesis was that this motor protein could therefore have a function in the neurophysiology of invertebrates and therefore in the head of *Drosophila melanogaster*. As the functions of myosin V in the cell are closely involved with its cargoes, we sought to isolate and

identify binding partners of myosin V in the head of *Drosophila* in order to elucidate possible roles for this molecular motor in the nervous system of this invertebrate species. *Drosophila* myosin V copurified with the photoreceptor-specific arrestin homolog, phosrestin-I. Arrestins are involved in the inactivation of many G protein-coupled receptor signaling cascades as well as desensitization of the ligand-activated state of the β-adrenergic receptor in non-retinal tissues. In the process of phototransduction in invertebrate retinal photoreceptor cells, light-activated rhodopsin, a G protein-coupled receptor, triggers the activation of phospholipase C (PLC), which in turn hydrolyzes the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) into the intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). Inositol triphosphate mobilizes intracellular calcium stores and opens cation-selective ion channels to generate a receptor potential and DAG acts through protein kinase C (PKC). Phosrestin-I binds specifically to the rhabdomeric membrane in a light-dependent manner and becomes phosphorylated by calmodulin-dependent kinase II (CaMKII) upon illumination. This is the first detectable phosphorylation event due to the action of a calcium/calmodulin dependent protein kinase in response to light in the *Drosophila* photoreceptor. Phosrestin -I facilitates the inactivation of metarhodopsin, the light-activated state of rhodopsin [11][12][13].

METHODS

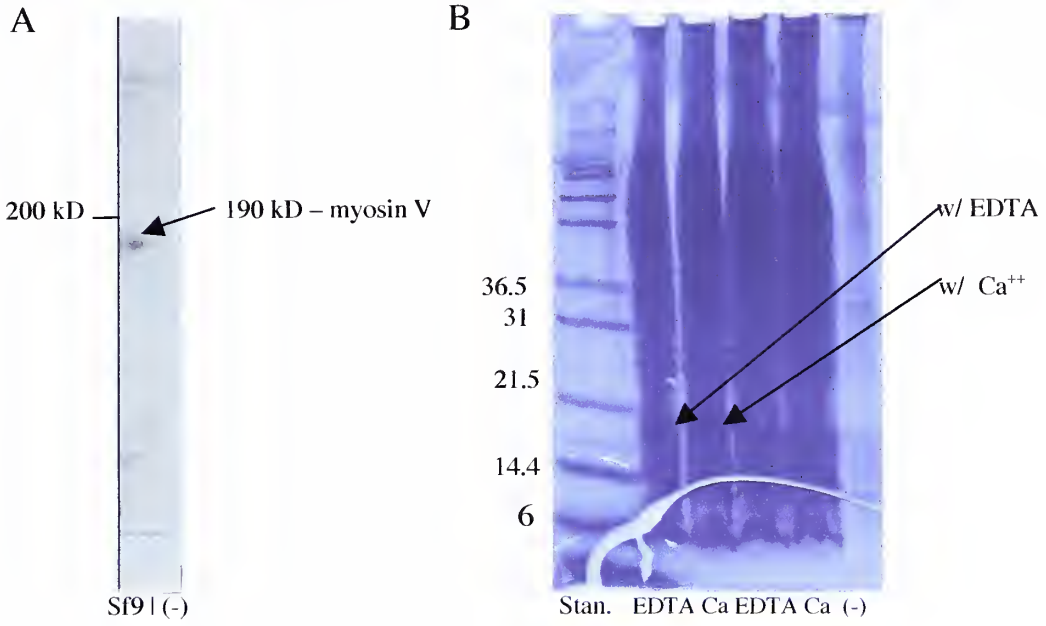
All studies described, except liquid chromatography-mass spectrometry, were performed by Erik D. Weiss.

Baculovirus transfer vector transfection

Construction of *Drosophila* myosin V-like fragment and its insertion into baculovirus transfer vector was carried out previously in this laboratory.

Baculotransfer vectors containing the desired cDNA for *Drosophila* myosin V heavy chain (dMV-HC), *Drosophila* calmodulin (dCaM), and dynein light chain (dynLC) are transfected into Sf9 insect cells by BaculoGold DNA (Pharmingen, San Diego, CA.). In order to confirm the success of our dMV-HC cDNA transfection, an extract from transfected Sf9 cells (3 days after transfection) is assessed by Western blot with a polyclonal antibody to myosin V (Figure 4A). In order to confirm the success of our dCaM cDNA transfection, an extract from transfected Sf9 cells (3 days after transfection) is size fractionated on a 10% SDS-PAGE gel in the presence of CaCl or EDTA. Calmodulin (~ 17 kD) is of higher molecular weight when complexed with calcium than without calcium, in the presence of the calcium chelator EDTA (Figure 4B). The resulting baculovirus is plaque purified and amplified to a titer of 5 X 10^8 to 2 X 10^{10} plaque-forming units/ml, and stored at 4°C as a stock solution. Sf9 cells (~1 L culture) are coinfected by the amplified dMV-HC, dCaM, and dynLC for three days. After collection, cell pellets are frozen in liquid nitrogen and either used immediately or stored at –80 °C.

Figure 4. Western blot using polyclonal antibodies to myosin V demonstrates expression of myosin V (~190 kD) in transfected Sf9 cells collected at 3 days (Figure 4A); Calmodulin shift assay demonstrates calmodulin expression in transfected Sf9 cells collected at 3 days (Figure 4B).



Affinity Purification

Experiments involve binding of dMV-HC, dCaM, and dynLC to the Anti-FLAG M2 Antibody Affinity Resin (Sigma-Aldrich, St. Louis, MO.). The charged resin is then incubated with a *Drosophila* head extract and washed. Myosin V, together with putative binding partners, are eluted using excess FLAG peptide and analyzed by SDS-PAGE and Western blot.

Once thawed, cell pellets are extracted at 4°C using 10 mM MOPS (pH 7.0), 0.2 M NaCl, 2 mM ATP (added after homogenization), 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/L leupeptin, and 1 mM dithiothreitol (DTT) and homogenized in a ground glass homogenizer. The mixture is sedimented at 47,000 X g for 15 min. The supernatant is passed through 500 µl (settled volume in column) Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) three times to optimize binding of

the FLAG-tagged myosin V to the affinity gel. The gel is washed once in 1 ml buffer A (0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF) and once in 1 ml buffer A plus 1 mM ATP and 5 mM $MgCl_2$. The gel is then washed with 1 ml buffer A alone, followed by a wash with 1 ml buffer B (10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF), followed by another wash with 1 ml buffer A and a succeeding wash with 1 ml buffer B. A final set of washes with 1 ml buffer A and 1 ml buffer B is carried out. The wash with MgATP helps to remove actin and the alternation of high and low ionic strength washes facilitates the removal of a FLAG antibody cross-reactive protein of about 110 kDa. Should this cross-reactive protein have remained in the final evaluated solution, its molecular weight difference from the 190 kDa myosin V would be easily distinguished.

One hundred fifty dry ice-sacrificed wild-type *Drosophila melanogaster* are decapitated by a graduated filter series mechanism. Heads are pooled, separated from abdominal, leg, and wing components under light microscopic examination, and lysed in 6 ml lysis buffer containing 80 mM NaCl, 20 mM Tris pH 7.5, 0.75% NP-40 (Pierce Chemical, Rockford, IL.), 100 μM guanosine-5'-O-(3-thio)triphosphate (GTPγS) (Sigma-Aldrich), 0.3 mM $MgCl_2$, 0.5 mM DTT, and a protease inhibitor mix (Roche Applied Science, Indianapolis, IN.) by brief homogenization in 1 ml homogenization tubes at 4°C. After centrifugation at 15,000 X g for 15 min at 4°C, 1 ml of lysate is incubated with myosin V-charged affinity resin by passing the lysate through the microcolumn three times.

The resin is washed at 4°C with 7 ml of wash buffer containing 80 mM NaCl, 20 mM Tris pH 7.5, 0.1% NP-40 (Pierce Chemical), 30 μM GTPγS (Sigma-Aldrich), 0.3

mM μl $MgCl_2$, and 0.5 mM DTT. dMV-HC, along with any interacting proteins, are eluted from the washed resin with 400 μL per 300 μl of settled resin, using elution buffer containing 200 mM NaCl, 10 mM Tris pH 7.5, 0.1 mM $MgCl_2$, and 0.4 mg/ml purified FLAG peptide. Eluate is collected in 0.1 ml fractions. Elution fractions are mixed with 20 μl 5X SDS sample buffer containing 10% SDS, 0.15 M Tris pH 7.5, 20 mM EDTA (pH 8.0), 20 mM β-mercaptoethanol, and 10% sucrose, and boiled for 2 min.

SDS-PAGE, Western Blotting, liquid chromatography-mass spectrometry

To look for dMV-HC as well as any putative interacting proteins in eluates, samples are size fractionated on 4-20% SDS-PAGE gels and stained with coomassie blue or transferred to nitrocellulose using a semidry blotter (model Trans-Blot SD; Bio-Rad, Hercules, CA.). Blots are probed with either polyclonal antibodies to MV or monoclonal antibody to the FLAG peptide. The resulting coomassie-stained polyacrylamide gel is analyzed by liquid chromatography-mass spectrometry (LC-MS) (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH) Separation of peptides and proteins occurs by reversed-phase liquid chromatography and the resulting gas-phase ions, not the precursor liquid droplets, are sampled by the MS apparatus. Mass spectrometry is a technique by which such gas-phase ions from intact, neutral molecules, are analyzed for molecular weight and sequence. This technique was utilized to determine the identity of the peptides and proteins eluted from our affinity purification and size fractionated on SDS-PAGE gels [8][14].

RESULTS

After elution from the Anti-FLAG M2 Affinity Gel with a solution containing excess FLAG peptide, products of the affinity purification are collected as eight 100 µl elution fractions. To look for dMV-HC as well as any putative interacting proteins in eluates, the 100 µl elution fractions are each mixed with 20 µl 5X SDS sample buffer, boiled for 2 minutes, and 20 µl from the eight 100 µl eluation fractions with SDS are size fractionated on 4-20% SDS-PAGE gels and stained with coomassie blue. Coomassie staining of polyacrylamide gels demonstrated protein bands at ~80 kD, 55.4 kD, 50 kD, 45 kD, and 21.5 kD (Figure 5).

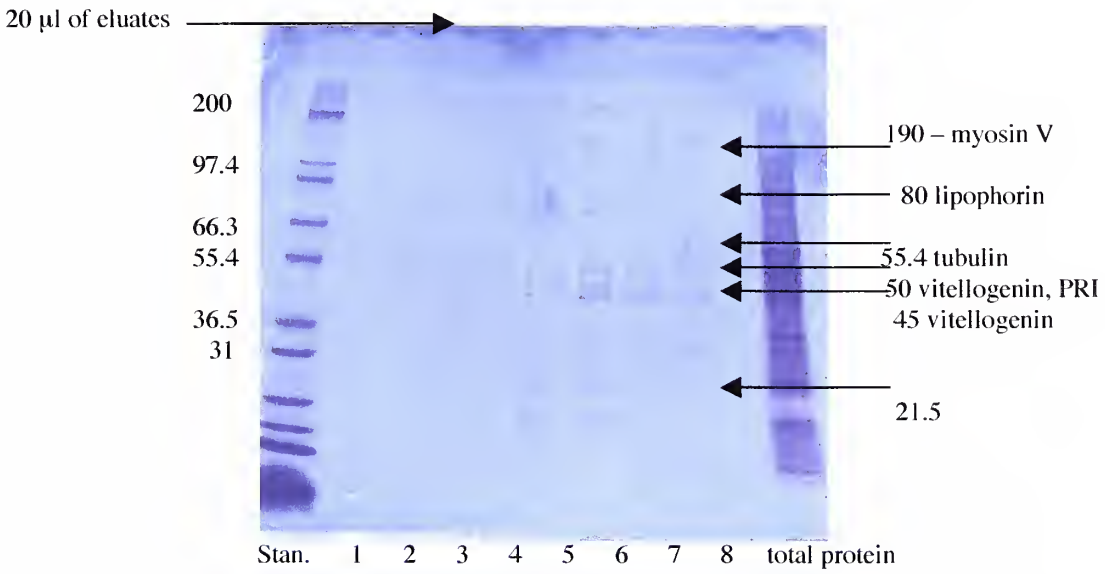


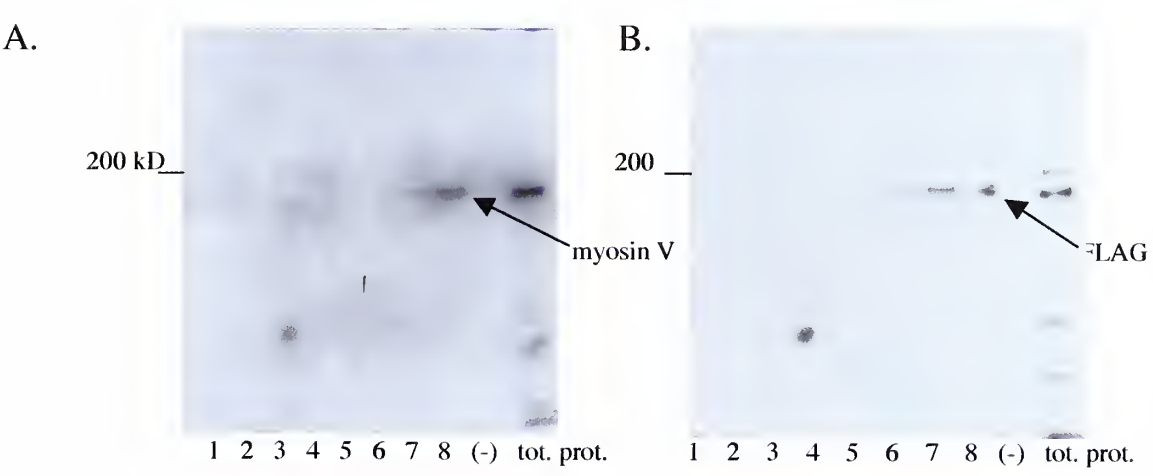
Figure 5. Polyacrylamide gel demonstrating coomassie staining of elution fractions from affinity purification, 20 µl of boiled (2 min.) solution containing 100 µl elution fraction and 20 µl 5X SDS sample buffer, were loaded in each lane well.

The band at 190 kD corresponded to the bait protein, myosin V. Bands at ~ 80 kD, 55.4 kD, 50 kD, 45 kD, and 21.5 kD appeared most reliably in fractions 4-8, indicating increased concentrations of these entities in the later stages of elution. Later analysis

probed these bands in as many fractions as possible within the parameters of liquid chromatographic resolution.

Western blot analysis of gels loaded with 20 µl of boiled (2 min.) solution containing 100 µl elution fraction and 20 µl 5X SDS sample buffer with a polyclonal Ab to myosin V (Figure 6A) and a monoclonal Ab to the FLAG peptide tag (Figure 6B) assured that the bait protein was indeed eluted with the putative binding partners. Myosin V, along with FLAG tag, eluted most reliably in fractions 7-8, indicating higher affinity binding of dMV-HC to Anti-FLAG M2 Affinity Gel than the putative binding partners. Coomassie stained polyacrylamide gels appeared to detect lower protein concentrations than the threshold of our myosin V polyclonal and FLAG peptide monoclonal antibodies, explaining the appearance of 190 kD myosin V bands in earlier (4-8) elution fractions on coomassie-stained gels (Figure 5).

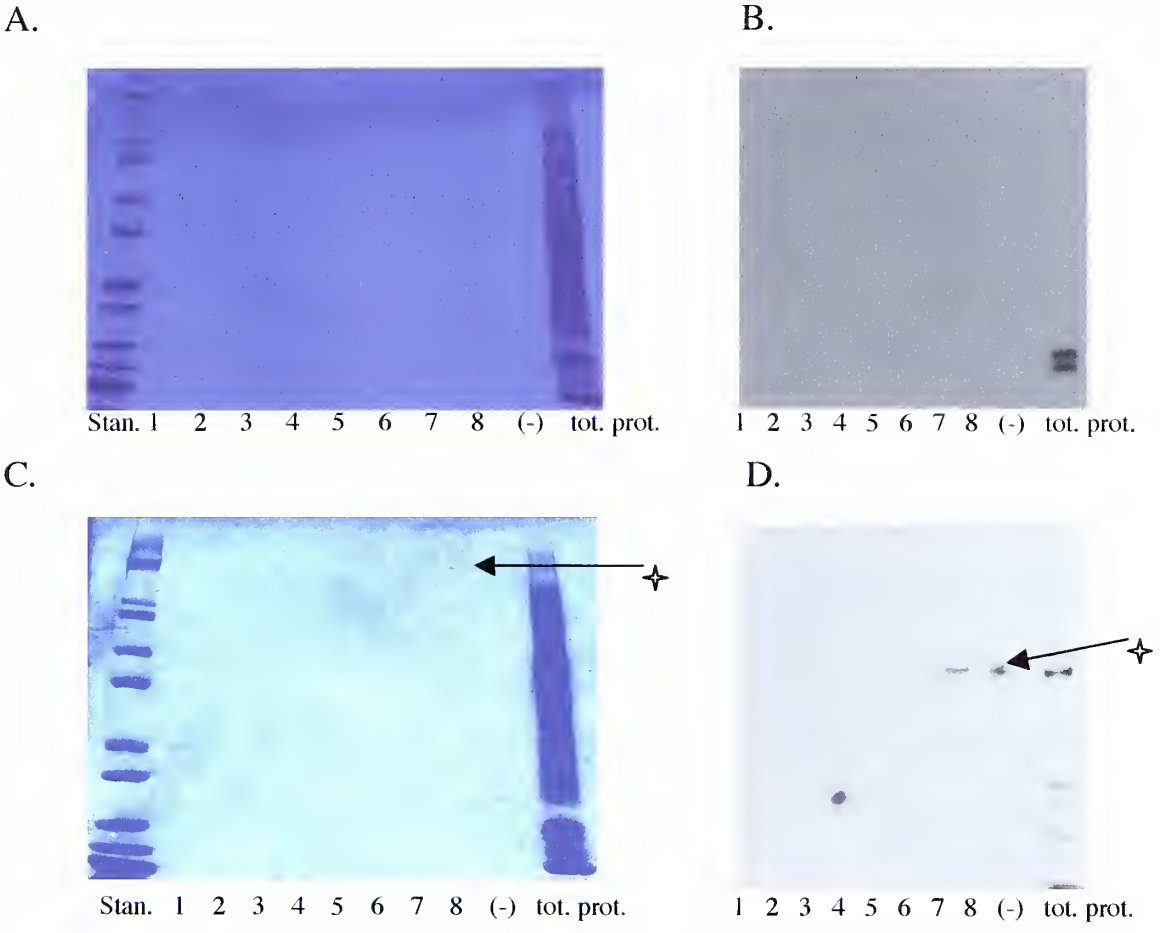
Figure 6. Western Blots of size-fractionated elution fractions using A) MV Ab and B) FLAG Ab, demonstrating the presence of the myosin V bait protein in the elution fractions resulting from the affinity purification.



Protein bands correlating to putative myosin V binding partners identified previously did not appear on SDS-PAGE gels from experiments conducted with cells that had not been

infected with virus expressing recombinant myosin V (Figure 7A) and in those without *Drosophila* heads in the extraction buffer (Figure 7C). The latter did stain for the 190-kD myosin V, the bait protein. Western blotting with monoclonal FLAG Ab demonstrated no bands in blots from experiments conducted with cells that had not been infected with virus expressing recombinant myosin V (Figure 7B) and the 190-kD myosin V band on blots from experiments conducted without *Drosophila* heads in the extraction buffer (Figure 7D). These control experiments demonstrated that the affinity purification of putative binding partners of myosin V along with the myosin V bait protein could only be achieved in the combined presence of myosin V-FLAG bound to Anti-FLAG M2 Affinity Gel and the *Drosophila* head extract. Elution from the Anti-FLAG M2 Affinity Gel alone incubated with *Drosophila* head extract or of Anti-FLAG M2 Affinity Gel to which myosin V-FLAG had been bound and incubated with extraction buffer without *Drosophila* heads do not yield these putative binding entities.

Figure 7. Polyacrylamide gel (A) and Western blot with FLAG Ab (B) from experiments conducted without virus. The low mol. wt. bands in (B) are likely degradation products of the FLAG antibody cross-reactive protein of about 110 kDa. Polyacrylamide gel (C) and Western blot with FLAG Ab (D) from experiments conducted with extraction buffer but without fly heads, demonstrating only the 190-kD myosin V band (+).



Bands on polyacrylamide gels were analyzed by LC/MS and found to contain lipophorin (80 kD band), vitellogenin I, II (50 kD band), and III (45 kD band) precursors, tubulin α-1 and β-1 chains (55.4 kD band), actin (45 kD band), ribosomal proteins (21 kD band), and phosrestin-I (50 kD band). Thus, we have identified a putative myosin V binding partner, phosrestin-I, in the head of *Drosophila melanogaster* by affinity purification.

DISCUSSION

PHOSRESTIN-I AND INVERTEBRATE PHOTOTRANSDUCTION

We have identified a putative myosin V binding partner in the head of *Drosophila melanogaster* by affinity purification. In addition to phosrestin-I, lipophorin, a shuttle for fat stores in the insect hemolymph, and vitellogenin, which is involved in the development of the female gonad, copurified with myosin V. These latter two proteins, along with tubulin, actin, and ribosomal proteins, are thought to be artifactual findings in the affinity purification considering their abundance in the hemolymph and lack of specificity to the insect head [15][16][17].

Phosrestin-I is a photoreceptor-specific protein and its association with myosin V would be of considerable interest since myosin V has been localized to rod photoreceptor synapses of the rat. Brain myosin V is distributed in several layers of the rat retina. There is intense myosin V staining particularly in the outer plexiform layer rod photoreceptor synapses, suggesting a role in visual processing. A synaptic myosin could be involved in the actin-dependent reorganization of photoreceptor synapses, such as that described in fish retinas in response to light. An actin-myosin V contractile mechanism may be involved in the plasticity of the photoreceptor synapse and the actin and myosin present in dendritic spines could provide a structural explanation for synaptic plasticity in general. This finding may be relevant to the neurological dysfunction found in *dilute* mutant mice as well as in the neurological deficits observed in patients with Griscelli syndrome [13].

The response to light stimulation in retinal photoreceptors involves the initiation of a G protein-coupled signaling cascade. Various G proteins are known to activate or inhibit adenylyl cyclase, cGMP phosphodiesterase, or phosphoinositide-specific

phospholipase C (PI-PLC). The receptor subgroups activating PI-PLC include: the α_1-adrenergic receptor, M_1 and M_3 muscarinic receptors; 5-HT_{1c} and 5-HT_2 serotonergic receptors; tachykinin receptors; prostaglandin F receptor; thromboxane receptor; certain classes of endothelin receptors; angiotensin II receptor; and invertebrate rhodopsin. In invertebrate light response, the light-dependent excitation of photoreceptors leads to activation of PI-PLC by rhodopsin [18].

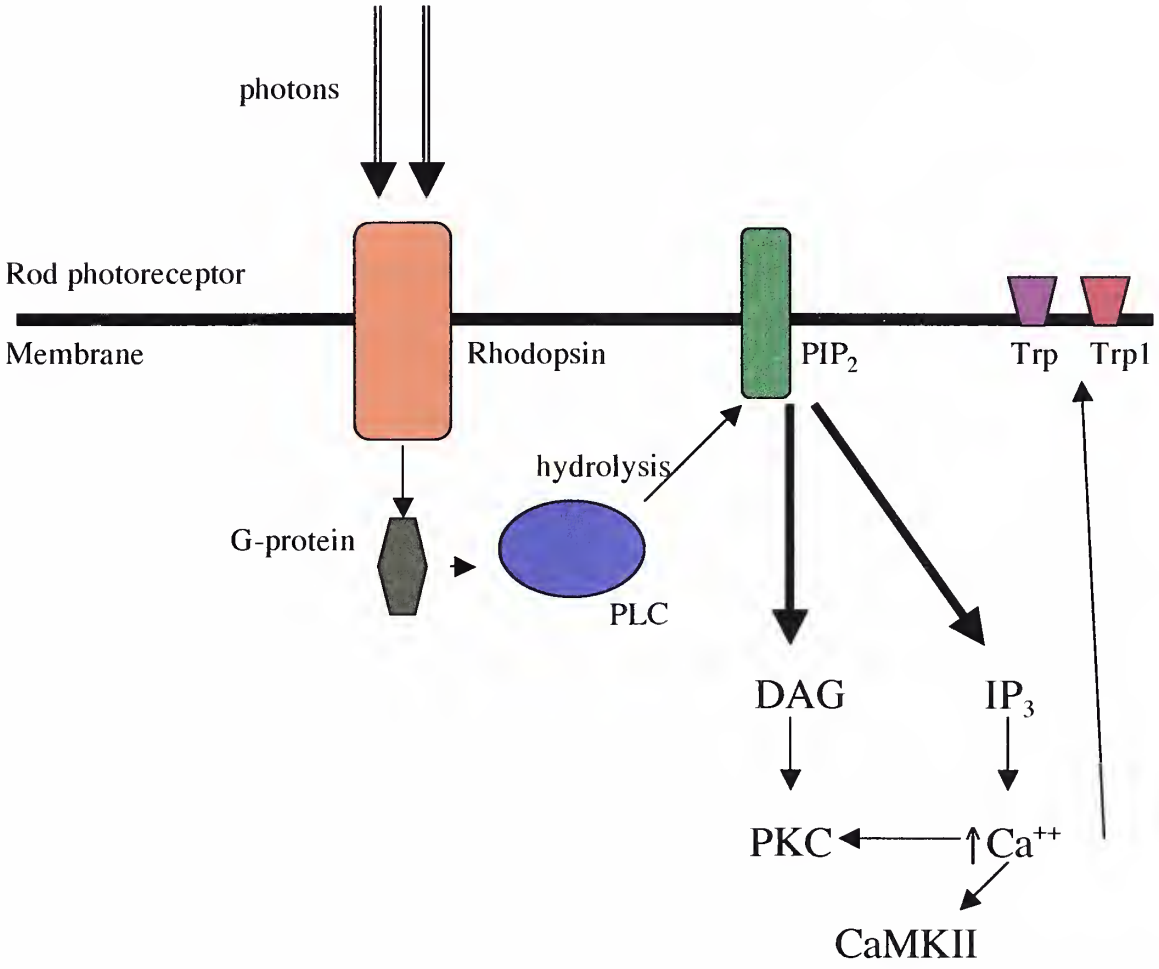


Figure 8. Invertebrate light activation through stimulation of rhodopsin and its associated G-protein signaling cascade.

Invertebrate rhodopsin consists of the apoprotein opsin, linked covalently to a chromophore, 3-hydroxy, 11-*cis*-retinal. Photon absorption leads to isomerization of 11-*cis*-retinal from 11-*cis* to all *trans*, producing a conformational shift resulting in activation of rhodopsin's catalytic properties. Photoactivated rhodopsin interacts with a photospecific G protein which in turn activates PLC. Phospholipase C hydrolyzes the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) into the intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). Inositol triphosphate mobilizes intracellular calcium stores, eventually leading to the opening of cation-selective ion channels (Trp and TrpI) resulting in the generation of a receptor potential. DAG acts through protein kinase C (PKC). Protein kinase C requires an increase in intracellular calcium for activation. Increased calcium also contributes to the activation of calmodulin-dependent protein kinase II (CaMKII) [11] [18] (Figure 8).

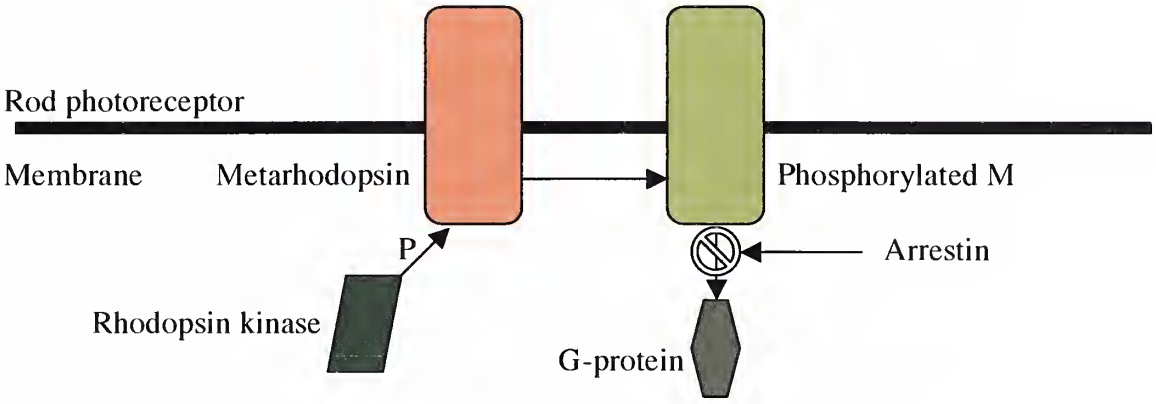
In vertebrates, unlike in the invertebrate system, the effector molecule of the phototransduction system is a cGMP phosphodiesterase (PDE). Activation of cGMP PDE leads to a transient decrease in intracellular cGMP. The vertebrate cation-specific channel is a cGMP-gated channel, which is open in the dark and closed in the presence of light and lowered cGMP levels. Activation of rhodopsin and the G protein, as well as the inactivation of the system, are fundamentally similar to the invertebrate system [19].

INACTIVATION OF PHOTOTRANSDUCTION

Metarhodopsin (M) is the light-activated state of rhodopsin, and its inactivation, or desensitization, in vertebrates is due to the action of at least two proteins, rhodopsin kinase and arrestin. In vertebrates, light-activated rhodopsin is phosphorylated by rhodopsin kinase at a cluster of serine-threonine residues at the COOH-terminal tail. The

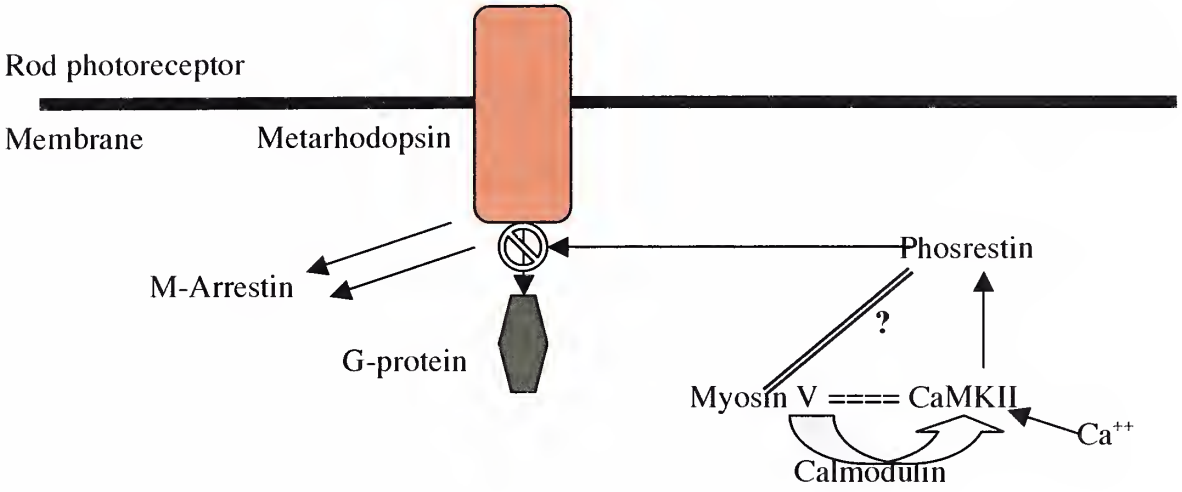
M phosphorylation minimally desensitizes the signaling cascade. Phosphorylation of the G protein-coupled receptor also increases its affinity for the arrestin class of cytosolic proteins, which terminates the activated state of the receptor signal by competing with the G protein for receptor binding [11][12] (Figure 9).

Figure 9. Vertebrate light inactivation by rhodopsin kinase and arrestin.



The termination of the active state of invertebrate rhodopsin is different from that of vertebrate rhodopsin, which requires phosphorylation of metarhodopsin. Inactivation of the active rhodopsin state in invertebrates results from a phosphorylated arrestin homolog binding to unphosphorylated metarhodopsin [21] (Figure 10).

Figure 10. Invertebrate light inactivation by phosrestin.



Arrestin, also known as soluble antigen or S antigen, was first identified as an abundant, soluble 48-kD protein in vertebrate retinal and pineal photoreceptors. Human and bovine arrestins have been linked to autoimmune disorders affecting vision. Functional analogs of the arrestins, known as β-arrestins, have been found in non-retinal tissues, and have been shown to inactivate the β-adrenergic receptor. In visual systems, arrestin proteins are predominantly located in the rhabdomeres, the specialized microvillar organelles containing the visual pigment rhodopsin and most of the phototransduction mediators [11]. A subclass of arrestins also has been shown to be involved in receptor-mediated endocytosis [12].

Drosophila has two photoreceptor-specific arrestin genes, arrestin 1 (Arr1) and arrestin 2 (Arr2). Arrestin 2, also known as phosrestin-I (PRI), is nearly 5-7 times more abundant in the rhabdomere than Arr1. Both Arr1 and Arr2 contribute to metarhodopsin inactivation. Consistent with its relative abundance, electrophysiological studies demonstrate that Arr2 is the primary inactivator of metarhodopsin. It inactivates M

through high-affinity 1:1 stoichiometric binding within milliseconds to create the inactive M-Arr2 complex [20].

Since invertebrate arrestin homologs are photophosphorylated, they are referred to as phosrestin I (PRI; 49K) and phosrestin II (PRII:39K). Phosphorylated PRI binds to rhodopsin and terminates the rhodopsin-activated G protein cascade, similar to the action of vertebrate arrestin [16]. The termination of the photoresponse in *Drosophila* occurs in less than 100 ms following termination of the light stimulus [19]. A PRI mutation producing a substantial reduction in PRI is characterized by a delayed deactivation in whole-cell voltage-clamp recordings. Thus, PRI seems to be required for the deactivation of the rhodopsin G protein-coupled signaling cascade [18].

Using whole cell patch-clamp recordings of the electrophysiological response of *arr2* loss-of-function mutant photoreceptors to light stimulation has demonstrated a large decrease in the rate of deactivation of the light response (166.9 ± 28.1 ms as opposed to 18.8 ± 2.9 ms for wild-type) defined as the recovery of the light response after termination of the stimulus. Unlike this response in *arr2* mutants, the rate of photoreceptor cell deactivation in *arr1* mutants is similar to that of wild-type controls. Thus, loss of Arr1 protein represents the loss of only a small fraction of the total arrestin function in inactivating metarhodopsin. Mutations in Arr1 have no identifiable phenotype. Loss-of-function mutations in Arr2 have defective rhodopsin inactivation and undergo light-dependent retinal degeneration [9] [11].

CALMODULIN-DEPENDENT PROTEIN KINASE II

Phosphorylation of PRI, at Ser_{366}, is due to the CaMKII pathway, not the PKC pathway, as evidenced by studies of fly mutants with defective PKC (Figure 10).

Phosrestin I has been shown to undergo light-induced phosphorylation by CaMKII faster than any other protein *in vivo* (400 ± 50 ms). The invertebrate phototransduction cascade leads as well to an increase in intracellular calcium. It was previously thought that the calcium- and light-dependent phosphorylation of PRI served as the signal for binding and inactivation of metarhodopsin. However, unphosphorylated PRI also has been shown to bind activated rhodopsin [12] [18].

Calmodulin-dependent protein kinase II associates with the actin cytoskeleton, synaptic vesicles, and postsynaptic densities, and is involved in neurotransmitter synthesis/release, regulation of ion channels, and long-term potentiation. In the pre-synaptic region, CaMKII regulates the interactions between synapsin I and synaptic vesicles and the actin cytoskeleton, which is a process involved in neurotransmitter release. CaMKII associates with postsynaptic densities, which are submembranous actin cytoskeleton structures postulated to regulate postsynaptic receptors and synaptic plasticity. The autophosphorylation of CaMKII affects its interaction with these postsynaptic densities. The kinase activity of CaMKII is regulated by the Ca^{++}-dependent binding of calmodulin [4].

Myosin V (MV) can bind and deliver calmodulin to CaMKII. CaMKII coimmunoprecipitates with MV from an extract of synaptosomes and has been found to bind the whole tail, medial tail, and proximal tail of MV, but not its head domain or C-terminal globular tail domain. The autophosphorylating activity of CaMKII and the substrate phosphorylation of MV take place within this complex. Thus, MV binds to CaMKII and activates its kinase activity in a Ca^{++}-dependent fashion. An hypothesis for this interaction is that in the presence of elevated intracellular free calcium, calmodulin

may translocate from MV to CaMKII, leading to the autophosphorylation of the latter and the subsequent phosphorylation of its substrate entities, such as phosrestin-I. [4] Calcium influx activates CaMKII, which phosphorylates the arrestin, and the product of the retinal degeneration C gene, rdgC, which dephosphorylates rhodopsin in vertebrate systems. Phosphorylating PRI makes it less basic and dephosphorylating rhodopsin makes it less acidic. Both of these steps are necessary for the interaction between rhodopsin and the arrestin in invertebrates [19].

RHODOPSIN-ARRESTIN COMPLEXES LEAD TO APOPTOSIS

Photoreceptors are lost through apoptosis in most of the common inherited degenerative diseases of the visual system that lead to blindness in humans, including retinitis pigmentosa, age-related macular degeneration, cone dystrophy, and Oguchi's disease [20]. Retinitis pigmentosa affects approximately 1 in 3000 people, can be inherited in an autosomal dominant, autosomal recessive, or X-linked fashion, and produces an early onset of night blindness leading to progressive vision loss, retinal degeneration due to apoptotic cell death, and blindness by middle age [19].

Light-independent photoreceptor degeneration is due to mutations in genes required for biosynthesis and maturation of rhodopsin or for the normal structure of the rhabdomere, the microvillar organelle which serves as the site for phototransduction. Light-dependent photoreceptor cell death may result from mutations in visual signaling molecules, such as rhodopsin, the Trp ion channel, arrestin 2, and RdgA diacylglyercol kinase, which lead to excessive activation of the phototransduction system resulting in a rapid necrotic death of photoreceptor cells. This death is likely due to the elevated intracellular calcium levels resulting from unregulated signaling activity [20].

Studies in a loss-of-function allele in the retinal degeneration C gene ($rdgC^{306}$) have demonstrated that visual arrestin (Arr2) also facilitates apoptosis of photoreceptor-containing cells following internalization of long-lived phosphorylated M-arrestin complexes. Arr2 is required for this apoptosis. The formation of stable M-p-arrestin complexes may be coupled with the clathrin-dependent internalization of these complexes from rhabdomeric membranes. [20] Stable, persistent complexes of rhodopsin and arrestin, which trigger apoptotic cell death, have been shown in several retinal degeneration mutants. Removal of either rhodopsin or arrestin reduces complex formation and rescues the degeneration phenotype. Retinal degeneration, moreover, requires the endocytic process. Thus endocytosis of rhodopsin-arrestin complexes is integrally involved in retinal degeneration [19].

In *Drosophila* photoreceptors, the primary mechanism for stabilizing metarhodopsin is the direct binding of arrestin. The average lifetime of metarhodopsin in the absence of bound arrestin is less than 2 min. The binding of metarhodopsin by arrestin increases its lifespan by at least two orders of magnitude. Upon light activation, Arr2 and metarhodopsin are cointernalized by an endocytic process, apparently to an internal compartment. Once internalized, these complexes are potentially stable for days. Arrestin also has been shown to be involved in clathrin and AP-2-dependent internalization of activated G protein-coupled receptors. Arr2 binds to clathrin in a phosphorylation-dependent manner. Transport of the Arr2-metarhodopsin complex from the rhabdomere to the cytoplasmic compartment is likely due to clathrin-dependent endocytosis. Arr2 could serve as an adaptor protein for this internalization [19].

Therefore, phosphorylation of metarhodopsin leads to apoptosis through an unknown mechanism downstream of the internalization process. RdgC phosphatase desphosphorylates metarhodopsin, counteracting this process. Phosphorylation of Arr2 suppresses apoptosis by disrupting clathrin interaction, thus preventing internalization of Arr2-metarhodopsin-p complexes. Activity of RdgC phosphatase and phosphorylation of Arr2 are upregulated by the light-induced increase in intracellular calcium. Phototransduction promotes the survival of photoreceptor cells by decreasing the accumulation of internalized Arr2-metarhodopsin-p complexes [20].

CONCLUSION

Myosin V has been shown to play diverse and important roles in vesicle and organelle trafficking along the actin cytoskeleton. Our preliminary findings showing copurification of phosrestin I with myosin V indicate a possible important role for myosin V in the modulation of light transduction. Phosrestin I is an invertebrate arrestin homolog involved in inactivation of the signal transduction triggered by the G protein-coupled receptor, rhodopsin, in response to light. Furthermore, previously shown interactions between arrestin and CaMKII and myosin V and CaMKII as well as the involvement of both arrestin and the cytoskeleton in clathrin and AP-2-dependent internalization support the potential importance to this finding of a myosin-arrestin connection.

Experiments designed to confirm this finding, such as coimmunoprecipitation, along with studies exploring the nature of the structural and functional interaction between myosin V and phosrestin-I, are needed to elucidate this possibly intriguing role for myosin V in the light-induced signaling cascade in insect photoreceptors. At a larger

level, these results may serve to expand our knowledge of the role of cytoskeletal motors, and the cytoskeleton itself, in intercellular signal transduction cascades. '

Glossary of Terms

Actin - thin filamentous track to which myosin motors transiently bind.

Adenylyl cyclase - enzyme that synthesizes cAMP from ATP. A second messenger relaying extracellular signals to intracellular effectors such as protein kinase A.

Af-6/*canoe* - family of proteins, first identified as an ALL-1 fusion partner in some patients with acute lymphoblastic leukemia, that has homology to the myosin V tail domain. In *Drosophila*, *canoe* interacts genetically with *Notch* and *Ras* signaling pathways.

AP-2 - heterotrimeric clathrin adaptor protein that mediates the internalization of plasma membrane proteins.

Arrestin - abundant, soluble, 48-kD protein in vertebrate retinal and pineal photoreceptors which terminates signaling by the activated, phosphorylated state of the G protein-coupled receptor, rhodopsin, by competitive inhibition, thus terminating the phototransduction signaling cascade.

ATP - adenosine triphosphate. Known as the "molecular currency" of intracellular energy transfer, ATP is able to store and transport chemical energy within cells. Hydrolyzed by myosin to adenosine diphosphate (ADP) and phosphate to generate force.

CaMKII - calmodulin-dependent protein kinase II. Kinase dependent on presence of calmodulin, which associates with the actin cytoskeleton, synaptic vesicles, and postsynaptic densities, and is involved in neurotransmitter synthesis/release, regulation of ion channels, and long-term potentiation. In the process of phototransduction, CaMKII phosphorylates phosrestin-I, enabling the inactivation of rhodopsin-mediated signaling by this invertebrate arrestin homolog.

cGMP - cyclic guanosine monophosphate. A cyclic nucleotide derived from guanosine triphosphate (GTP), cGMP acts as a second messenger, most notably by activating intracellular protein kinases in response

to the binding of membrane-impermeable peptide hormones to cell surface receptors.

cGMP phosphodiesterase - enzyme catalyzing the hydrolysis of phosphodiester bonds, as in a molecule of cGMP, converting it to GTP.

Calmodulin - ubiquitous calcium-sensing protein. A small, acidic protein (~ 148 amino acids), which contains four EF-hand motifs, each of which binds a calcium ion. A key component of the calcium second messenger system, involved in controlling many of the biochemical processes of cells. Highly conserved among eukaryotes.

Calmodulin/EF-hand proteins – superfamily of calcium-binding proteins that make up myosin regulatory light chains. Other members of the EF-hand family include aequorin, calbindin, calcineurin, calpain, fimbrin, osteonectin, spectrin, and troponin C, among others.

Clathrin - major protein of the polyhedral coat of coated pits and vesicles involved in endocytosis. Clathrin-coated pits move in the plane of the membrane and are tethered to cytoskeletal elements. Once the internalized vesicle is formed, the clathrin coat is lost and the vesicles form endosomes or receptosomes.

Conventional myosins – myosins with tail domains that dimerize through the formation of coiled-coil structures and assemble to form filaments.

DAG - diacylglycerol. Intracellular messenger which acts through protein kinase C (PKC)

dilute - mouse gene which encodes the myosin Va heavy chain. Mutations associated with dilute or pale coat color.

Dynein light chain - light chain from the minus-end-directed microtubule motor, dynein.

EF-hand - a common calcium-binding motif.

Eukaryote - organism consisting of one or more cells containing membrane-bound nuclei, as well as organelles.

Griscelli disease - human myosin V mutation-associated disease. Autosomal recessive condition characterized by pigmentary dilution, silvery hair, pigment aggregates in hair shafts, accumulation of melanosomes in melanocytes, variable cellular immunodeficiency, and hemophagocytosis.

Hemolymph - blood analogue used by those animals, such as all arthropods, that have an open circulatory sstem. In these animals there is no distinction between blood and interstitial fluid. The liquid fills all of the interior of the body and surrounds all cells.

Invertebrate - any animal without a backbone or vertebra (ie. insects, squids, or worms)

IP_3 - inositol triphosphate. Intracellular messenger which mobilizes intracellular calcium stores, eventually leading to the opening of cation-selective ion channels.

IQ motifs - myosin neck domain light-chain binding regions with conserved isoleucine and glutamine residues.

Keratinocyte epidermal cell (skin) that produces keratin.

Lipophorin - shuttle for fat stores in insect hemolymph.

M - metarhodopsin. Light (photon)-activated state of rhodopsin.

Melanocyte – located in basal cell layer of skin, produces melanin (brown pigment) within melanosomes and tranfers these organelles to keratinocytes.

Melanosome - organelle in melanocyte within which melanin is produced. Melanocytes transfer melanosomes to keratinocytes.

Myosin – superfamily of molecular motors that hydrolyze ATP to generate energy and use actin filaments as tracks for unidirectional force production.

MV - myosin V. Unconventional myosin class that serves as a processive motor in vesicle/organelle transport. Binds CaMKII and activates its kinase activity in a Ca^{++}-dependent fashion.

Photoreceptor

cells - cells on retina of eye which contain pigment molecules that absorb light.

PI-PLC - phosphoinositide-specific phospholipase C. Hydrolyzes the membrance phospholipid PIP_2 into the intracellular messengers IP_3 and DAG.

PIP_2 - phosphatidylinositol-4,5-bisphosphate. Membrance phospholipid which is hydrolyzed by PLC into the intracellular messengers IP_3 and DAG.

PRI - phosrestin-I. Photoreceptor-specific invertebrate arrestin homolog, which is phosphorylated by CaMKII and terminates signaling by the activated state of the G protein-coupled receptor rhodopsin by competitive inhibition.

Processive motor – molecular motor that undergoes multiple cycles of MgATP hydrolysis with movement along a filament before dissociation.

Prokaryote - single-celled organism without a nucleus.

Rab27a - member of the Rab family of small GTPases, involved in the melanosome capture mechanism in melanocytic dendritic processes. Rab27a enables myosin Va-dependent melanosome capture and recruits the myosin to the melanosome surface, perhaps acting at least in part as the melanosome receptor for myosin Va.

rdgC - retinal degeneration C gene. Gene product dephosphorylates rhodopsin in vertebrate phototransduction systems.

Retinitis pigmentosa - degenerative disease of the visual system which affects approximately 1 in 3000 people, can be inherited in an autosomal dominant, autosomal recessive, or X-linked fashion, and produces an early onset of night blindness leading to progressive vision loss, retinal degeneration due to apoptotic cell death, and blindness by middle age.

Rhabdomere - specialized microvillar organelle containing the visual pigment rhodopsin and most of the phototransduction mediators.

Rhodopsin - G protein-coupled receptor involved in the initiation of signaling in phototransduction. Invertebrate rhodopsin activates PI-PLC,

through a photospecific G protein, and consequent hydrolysis of PIP_2 to IP_3 and DAG, thus producing mobilization of intracellular calcium stores, opening of cation-selective ion channels, generation of a receptor potential, activation of protein kinase C (PKC), and activation of CaMKII.

Rhodopsin kinase - enzyme which phosphorylates metarhodopsin, the light-activated state of rhodopsin, in vertebrate phototransduction systems, minimally desensitizing the G protein-coupled signaling cascade and increasing the affinity of metarhodopsin for the arrestin class of cytosolic proteins.

Unconventional myosins – myosin proteins that do not have tail domains that dimerize and form filaments.

Vertebrate - any animal with a backbone or vertebra.

Vitellogenin - protein involved in the development of the insect female gonad. [22]

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